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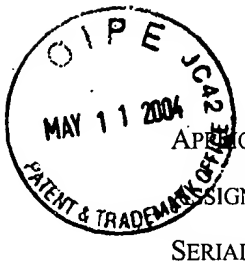

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Comb *et al.*		
ASSIGNEE:	CELL SIGNALING TECHNOLOGY, INC.		
SERIAL NUMBER:	09/535,364	EXAMINER:	P. Ponnaluri
FILING DATE:	March 24, 2000	ART UNIT:	1639
FOR:	PRODUCTION OF MOTIF-SPECIFIC AND CONTEXT-INDEPENDENT ANTIBODIES USING PEPTIDE LIBRARIES AS ANTIGENS		

May 11, 2004
Beverly, Massachusetts

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DECLARATION OF ROBERTO D. POLAKIEWICZ, Ph.D., UNDER 37 C.F.R. §1.132

I, Roberto D. Polakiewicz, do hereby declare and aver the following:

1. I am the Vice President of Research and a Senior Scientist at Cell Signaling Technology, Inc., the assignee of the above-identified patent application. I have held this position since November 1999.

2. I hold a Doctorate degree in Microbiology, which I received from the Hebrew University of Jerusalem in 1992. I also hold a Master of Sciences degree in Microbiology and a Bachelor of Science in Chemistry, both of which I received from the University in Jerusalem prior to my Doctoral studies.

3. Following completion of my Doctoral degree, I was a Post-doctoral Fellow at the Whitehead Institute for Biomedical Research (M.I.T.), and then a Staff Scientist at New England Biolabs, Inc., in Beverly, Massachusetts, from 1995 to 1999, during which time I conducted research in the area of signal tranduction. My years of research at New England Biolabs included, among other things, the production, characterization, and use of antibodies, including anti-peptide, phosphorylation-site specific antibodies, to study signal transduction pathways.

4. I have over nine years of research experience in the area of signal transduction cascades and pathways, including the production and use of anti-peptide antibodies specific for

protein phosphorylation sites relevant to these signaling pathways. My research experience includes examination of recurring phosphorylatable motifs, such as kinase consensus substrate motifs, that are relevant to signal transduction pathway research. I have authored or contributed to more than ten articles or papers relating to signal transduction research, most of which involve the production and/or use of phospho-specific anti-peptide antibodies.

5. I am, therefore, familiar with and skilled in the art of antibody production, characterization, and use, including the production, screening, characterization, and use of anti-peptide antibodies and phosphorylation site-specific antibodies. I am also familiar with and skilled in signal transduction pathway research and the production and use of antibodies to study such pathways, including the production and use of anti-peptide antibodies against phosphorylated protein sequences, sites, or motifs relevant to signal transduction.

6. I understand that the Examiner in the above-identified patent application has issued a Final Office Action, mailed November 12, 2003, rejecting the pending claims, which are directed to a novel class of motif-specific, context-independent antibodies that specifically bind small recurring , phosphorylated kinase consensus substrate motifs or protein-protein binding motifs in multiple different peptides or proteins in an organism in which the motif recurs. I further understand that the Examiner has expressed an opinion that the art of producing, characterizing, and using phospho-specific antibodies (including those of the present invention) is unpredictable. I also understand that -- based in part on this alleged unpredictability and in part on the proposition that the species of antibodies described in the Examples are not representative of the claimed genus -- the Examiner has expressed an opinion that one of ordinary skill in the art of antibodies would not recognize the inventors (of the present application) were in possession of the genus/class of antibodies as broadly as presently claimed (claim 28) (see November 12, 2003 Office Action at pages 9-10).

7. I have reviewed both the pending claims and the specification at issue (hereinafter referred to as the " '364 specification"), and the outstanding Office Action. I am aware of my duty of candor under 37 C.F.R. §1.56. I am also familiar with the standard technical references in the antibody field, including those cited by Applicant in, or in support of, the '364 patent application, as further discussed below.

8. Based on my familiarity with the field of antibodies (including their production and use in signal transduction research) it is my opinion that scientists of ordinary skill in this art (including anti-peptide, phospho-specific antibody production and use) would recognize -- based on the detailed written description provided in the '364 specification and the high level of knowledge in

this art -- that the inventors were in possession of the claimed genus of novel motif-specific, context-independent antibodies at the time the '364 application was filed. It is also my opinion that the art of antibodies, their production, characterization, and use (including anti-peptide, phospho-specific antibodies) is predictable and that the level of skill and knowledge in this mature art is high. As discussed in more detail below, the advanced knowledge in, and predictability of, this art are supported by several well-known, detailed technical references in the antibody production field, which establish standard and proven methodologies for the production, characterization, and use of antibodies, including anti-peptide antibodies.

9. Standard methods and techniques for the production, characterization, and use of antibodies with desired specificities are well developed and have been around for many years. These techniques are mature and well proven. For example, an entire seven-hundred page manual, ANTIBODIES: A LABORATORY MANUAL, Harlow & Lane (1988), Cold Spring Harbor Laboratory, is devoted solely to the production, characterization, and use of antibodies (three detailed chapters are dedicated to the structure, features, and characteristics of antibodies and their binding; four detailed chapters alone are dedicated to the production and screening of antibodies – including anti-peptide antibodies --; and another six detailed chapters are dedicated to their use). This manual is widely known and used by those of skill in this art and its techniques are considered standard. Another widely-known and standard reference in this art is CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Volume 2, John Wiley & Sons (1992), which devotes over 102 pages, in Section 11, solely to the production, characterization, and use of antibodies, including anti-peptide antibodies. Furthermore, proven and well-known techniques for the use of synthetic peptide antigens -- including phosphorylated peptide antigens -- to produce and characterize anti-peptide antibodies have been established for some time. For example, Czernik *et al.*, METHODS IN ENZYMOLOGY *201:* 264-283 (1991) and Czernik *et al.*, *Neuroprotocols 6:* 56-61 (1995) described and established the standard technique for producing, screening, and characterizing phosphorylation site-specific antibodies using synthetic peptide antigens. The general technique first described in Czernik has been practiced for more than a decade, and remains widely practiced today as the standard anti-peptide phospho-antibody production methodology. Indeed, this established methodology is cited in the Background section of the '364 specification, and was roughly followed and described in the '364 specification for producing the motif-specific, context-independent antibodies of the invention (excepting the inventors' novel modification of immunizing with a degenerate peptide library antigen (as opposed to a single peptide antigen)) to produce broadly-reactive, context-independent antibodies.

10. Subsequent improvements in the standard technique for anti-peptide, phospho-specific antibody production and characterization were later developed and become part of the

standard methodology at the time the '364 application was filed. For example, Bangalore /Stern *et al. Proc. Natl. Acad. Sci. 89:* 11637-11641 (1992) and Epstein *et al.*, U.S. Patent Number 5,599,681, "Activation State-Specific Phosphoprotein Immunodetection," Issued Feb. 4, 1997, each described the use of chemically (rather than enzymatically) phosphorylated amino acid residues in synthetic peptide antigens according to the standard methodology set forth by Czernik. This simplified practice became the standard anti-phosphopeptide antibody production technique, both at the time the present application was filed and to this day. Indeed, the number of scientific literature references describing the use of phospho-peptide antigens in the standard methodology to produce phosphorylation site-specific antibodies is too numerable to list here. The phospho-specific antibody research market itself is a mature one, estimated at around $70 million annually, and many commercial suppliers, including BioSource, Santa Cruz Biotechnology, Upstate Biotechnology, Becton-Dickinson, and Cell Signaling Technology (the assignee of the '364 application) develop and sell a great number of such antibodies.

11. The proven and widely-practiced methodology set out in the references discussed above teaches, in detail, (i) how to construct and immunize an animal with an appropriate peptide antigen encompassing a desired target epitope, (ii) how to screen produced antibodies to identify and isolate those with the desired specificity and binding qualities, (iii) how to characterize the antibodies, (iv) and how to use them in standard immunological assays to bind desired proteins or peptides. These standard and time-tested techniques have largely made the production of anti-peptide antibodies to a desired epitope straightforward and routine, and the ability to produce, screen, isolate and use anti-peptide antibodies with a desired specificity is therefore quite predictable. Those familiar with the standard techniques taught by such references and possessing ordinary skill in the art of antibody production and characterization have sufficient knowledge and skill to readily produce, screen, characterize, and use anti-peptide antibodies that specific for essentially any desired epitope, including modified (e.g. phosphorylated) sequences.

12. Building on the standard and mature techniques for producing anti-peptide, phosphorylation site-specific antibodies, the '364 specification describes, in detail, how to produce a novel type of anti-peptide antibody – called a "motif-specific, context-independent antibody," which is capable of specifically binding a short, recurring, modified target motif (*e.g.* a phosphorylated kinase consensus substrate motif) in multiple different peptides/proteins in which it recurs, by the novel modification of using a degenerate peptide library antigen (rather than the usual single peptide sequence antigen) for immunization. Otherwise, the methodology largely follows the standard protocols. The '364 specification describes, throughout, the importance of both the motif-specific *and* context-independent characteristics of the novel class of antibodies disclosed; indeed, the lack

of prior art antibodies having such qualities is described in the Background section of the '364 specification and is the very problem solved by the invention. Namely, the standard anti-phosphopeptide antibody production technique using many copies of only a single phosphopeptide sequence as an antigen, while time-tested and useful, does not reproducibly yield antibodies capable of binding many different proteins containing a recurring (and often degenerate) motif sequence. Rather, phosphorylation "site-specific" antibodies are produced that bind a single unique phospho-epitope typically found in a particular protein (such as exemplary phosphorylation site-specific antibodies described in Czernik, Epstein, and Bangalore/Stern). In contrast to the novel class of antibodies disclosed in the '364 application, such traditional site-specific antibodies only bind the particular target protein when phosphorylated at the unique site. Accordingly, site-specific antibodies are not useful for detecting *many* different proteins containing a recurring, modified motif (such as a phosphorylated kinase consensus substrate site). For example, the exemplary Synapsin I phosphorylation site-specific antibody described in Czernik only binds Synapsin I when phosphorylated at a unique 11-residue sequence. Similarly, the exemplary DARPP-32 phosphorylation site-specific antibody described in Czernik only binds DARPP-32 when phosphorylated at a unique 10-residue long sequence. Neither antibody is designed to, nor capable of, specifically binding a shorter recurring (and often degenerate) motif of the type relevant to signal transduction pathways, and to which the '364 application relates. The unmet need for better antibodies (having new binding qualities) to study such recurring motifs is clearly discussed in the Background of the '364 specification, and was solved by the inventors' creation of a novel class of "motif-specific, context-independent" antibodies.

13. The '364 specification describes, in detail, how modifying the standard anti-peptide methodology by the novel use of a degenerate peptide library antigen comprising many different peptide sequences having only the desired target motif in common (as opposed to the traditional single peptide sequence antigen), results in production of antibodies specific only for the motif that is common to (fixed in) all peptide sequences in the antigen, irrespective of the surrounding peptide context. *See* '364 specification at p. 12, lines 21-26. Generally following the standard anti-peptide methodologies known in the art, the '364 specification provides a detailed description of how to construct a synthetic peptide antigen (here, a degenerate peptide library) encompassing a desired target motif, and how to immunize an animal with the antigen in order to generate desired antibodies. *See, e.g.* '364 specification at pages 14-16. The '364 specification provides a detailed description of how to screen and select resulting antibodies having the desired specificity (for the target motif) and the desired characteristic of context-independence (*i.e.* ability to bind many different sequences containing the target motif) according to standard techniques. *See, e.g.* '364 specification at pages

16-18. The production of monoclonal antibodies is also described in detail. *See* '364 specification at pages 18-19. Thus, the '364 specification describes to one of skill in the art of antibody production, and familiar with the standard references and methodologies, how to produce, isolate, and characterize an antibody within the claimed genus.

14. The '364 specification describes in detail the types of modified residues, including phosphorylated residues, that may be included in the recurring target motif recognized by the context-independent antibodies of the invention. *See, e.g.* '364 specification at pages 7 and 15. The '364 specification describes, throughout (including its Examples), the types of short, recurring modified motifs against which context-independent antibodies may beneficially be produced. For example, kinase consensus substrate motifs (such as the CDK consensus motif) and protein-protein binding motifs (such as 14-3-3 binding motif) – and the need for context-independent antibodies capable of binding many different proteins in which such motifs recur -- are described as being of particular importance to signal transduction research. *See, e.g.* '364 specification at pages 1-2, 8, 19-20, 26. The structure and characteristics of such motifs, which recur in multiple different proteins and serve as conserved or "consensus" phosphorylation sequences for a particular enzyme (*e.g.* a MAPK kinase) or as conserved protein-protein binding sites (*e.g.* a 14-3-3 binding site), have been well described and are known to those of skill in the art of signal transduction research. *See, e.g.* Kemp *et al., Trends in Biochem. Sci. 15:* 342-46 (1990); Kemp *et al., Methods in Enzymology 200:* 62-81 (1991); Songyang *et al., Mol. Cell Biol. 16:* 6486-493 (1996); al-Obeidi *et al., Biopolymers 47:* 197-223 (1998); *see also* L. Cantley, overview in Cell Signaling Technology, Inc. 2000-2001 Catalogue at p. 198. Such motifs typically include two to six invariant residues including one or more modified residues, and often (but not always) one or more variable (or degenerate) positions. They recur *because* they are short, unlike longer, unique phosphorylation site sequences bound by site-specific antibodies, which are typically ten two fifteen or more fixed residues. Exemplary consensus phosphorylation motifs for various kinases are described, for example, in Table II in Kemp *et al.* (1991) and Table III in al-Obeidi *et al.*, copies of which are attached hereto for convenience, which tables are represented here for further convenience:

Exemplary Consensus Motifs (taken from Table II, Kemp et al.)

Calmodulin-dependent protein kinase II	XRXXS*/T*
Casein kinase I	S(P)XXS*/T*
Casein kinase II	S*/T*XXEX
c-AMP dependent kinase	RRXS*
c-GMP dependent protein kinase	R/KXS*/T*

Glycogen synthase kinase-3	S*XXXS(P)
Phosphorylase Kinase	K/RXXS*V/I
Tyrosine Kinase/EGFR kinase	XE/DY*X

Exemplary Consensus Motifs (taken from Table III, Al-Obeidi et al.)

PKA kinase	RRXS*
PKG kinase	RXXS*R
CK2 kinase	S*XXE/D
CamKII kinase	RXXS*V

Many other publications and reports describing such motifs are available in the scientific literature. Techniques for determining the sequence of a consensus motif for a particular kinase of interest are also well described. *See, e.g.,* Cantley et al., U.S. Patent No. 5,532,167, "Substrate Specificity of Protein Kinases" (Issued July 2, 1996); Yaffe *et al., Nat. Biotech 19:* 348-353 (2001), Tegge *et al., Biochemistry 34(33):* 10569-77 (1995); Tegge *et al., Methods Mol. Biol. 87:* 99-106 (1998). Thus, the '364 specification describes to one of skill in the art of antibody production, and familiar with the standard references and methodologies, how to produce, isolate, and characterize an antibody within the claimed genus that is specific for a desired motif.

15. The '364 specification also describes in detail how to use the powerful new class of motif-specific, context-independent antibodies in standard immunological formats and methods to rapidly detect many different proteins containing a target motif using a single antibody. For example, use of such context-independent antibodies to profile genome-wide changes in phosphorylation of proteins containing a particular motif (such as MAPK consensus site or cdc2 kinase consensus site) are described in detail. *See* '364 specification at pages 19-27.

16. The description of the claimed genus of antibodies that is provided in the '364 specification includes the experimental details of the actual production of eight exemplary motif-specific, context-independent antibodies that bind modified targets containing at least one phosphorylated amino acid. *See* '364 specification at pages 27-58. Of these, six examples (II-IV and VI-VIII) describe motif-specific, context-independent antibodies, within the presently claimed genus, that bind representative phosphorylated kinase consensus substrate motifs or protein-protein binding motifs ((namely, PXS*P (MAPK consensus substrate site), RSXS*XP (14-3-3 binding site), PXT*PXR (CDK consensus substrate site), RXRXXT* (AKT consensus substrate site), RRXT* (PKA consensus substrate site), and [F/Y][T/S]* or [T/S]*F (Bulky-ring directed kinase consensus

substrate site, where *=phosphorylated residue)). Clearly, the inventors had possession of these representative antibody species since they were actually produced and their binding specificity and characteristics described in detail. Moreover, these exemplary species, which bind phosphorylated kinase consensus substrate or protein-protein binding motifs consisting of one to four invariable residues, and including from zero to four variable (X) positions, are representative of the typical variation of motif species within the genus. These motifs have structures typical for kinase consensus substrate motifs and protein-protein binding motifs, which, as described above, are short, recurring sequence motifs that include one or more phosphorylatable amino acids (frequently serine or threonine), one or more required (or fixed) additional residues, and frequently include one or more non-required (degenerate or variable) positions. *See, e.g.* Kemp *et al.*, al-Obeidi *et al.*, *supra.*

17. It is my opinion, based on the detailed description of production, characterization, and use of the claimed class of novel antibodies provided in the '364 specification, coupled with the high level of skill and predictability in the art of anti-peptide antibody production, that the inventors were in possession of the claimed genus of antibodies as of the filing date of their application. A scientist familiar with the art of antibody production and signal transduction pathways could, following the description and methodology provided in the '364 specification, produce a context-independent antibody specific for any modified motif of interest, including phosphorylated kinase consensus substrate motifs or phosphorylated protein-protein binding motifs. The eight working Examples provided in the '364 specification evidence this. Indeed, to the best of my knowledge, information, and belief, CELL SIGNALING TECHNOLOGY, INC. (the assignee of the '364 application) has to date produced over 20 motif-specific, context-independent antibodies, including antibodies against at least 16 different phosphorylated kinase consensus substrate motifs and protein-protein binding motifs, and only a single attempt did not yield an antibody with the desired characteristics of motif-specificity and context-independence. Many of these antibodies are presently listed for sale in CELL SIGNALING TECHNOLOGY'S 2003-04 catalogue (at page 14; see attached). These results further evidence the predictability and reproducibility of the methodology described in the '364 specification and that the inventors were in possession of the claimed genus of antibodies, not merely the representative species they produced in the Examples.

18. Antibodies within the presently claimed genus of antibodies (*see* claim 28, pending) are specific for a kinase consensus substrate motif or protein-protein binding motif containing at least one phosphorylated amino acid, having up to four invariable amino acids and optionally, one or more degenerate positions, and specifically bind the motif (*i.e.* motif-specificity) in a plurality of peptides or proteins in an organism in which it recurs (*i.e.* context-independence). Those of skill in the art of antibody production and familiar with signal transduction pathways and research would

readily understand how the binding characteristics and specificity a motif-specific and context-independent antibody described and claimed in the '364 specification differ from the binding characteristics and specificity of traditional phosphorylation site-specific antibodies (such as those described, *e.g.* in Czernik). Is it my opinion, therefore, as one of skill in the art of antibody production, characterization, and use, and familiar with signal transduction pathways, that the inventors were in possession of the claimed genus of novel antibodies, which are distinct from prior art site-specific antibodies.

19. It is further my opinion that the six exemplary phosphorylated kinase consensus substrate motifs and protein-protein binding motifs described in Examples II-IV and VI-VIII of the '364 specification are representative of typical kinase consensus substrate motifs and protein-protein binding motifs relevant to signal transduction, and that the inventors of the '364 application were in possession, not only of these representative motif-specific, context-independent antibodies, but of the broader class of antibodies as presently claimed at the time the present application was filed.

20. I further understand that the Examiner has taken the position that the failure of a prior attempt to produce a phosphothreonine-specific antibody as reported in Heffetz *et al.* (discussed and distinguished in the Background section of the '364 specification, at page 5) indicates that the production of phospho-specific antibodies is unpredictable. This assertion is incorrect. First, as discussed in detail in the preceding paragraphs, the art of producing, screening, characterizing, and using anti-peptide antibodies is well established, proven, and predictable; the techniques have become routine to those of skill in the art. The eight Examples provided in the '364 specification further confirm the predictability and reproducibility of anti-peptide antibody production techniques, including the modified and novel anti-peptide production technique described in the '364 specification. Accordingly, the *single* failed attempt described in Heffetz in no way undermines the entire body of scientific literature and decades of experience indicating the mature, advanced, and predictable art of antibody production. *See* references cited and discussed above.

21. Secondly, the failed Heffetz attempt to produce a phosphothreonine-specific antibody is readily distinguishable because it the attempt did *not* use anti-peptide production techniques. Rather, it was an attempt to produce the desired antibody by coupling a phosphothreonine residue to a standard carrier protein, keyhole limit hemocyanin (KLH), in the hopes of raising an immune response directed only to the phosphoresidue. *See* Heffetz *et al.*, *Method. Enzymology 201:* 44 (1991), cited in '364 specification on page 5. The failure of such attempts were not surprising, since it is well known in the art that coupling a small chemical entity (a hapten) to a carrier, like KLH, may or may not produce antibodies against the small chemical entity. Indeed, the unpredictability

and unsuitability of hapten-carrier methods to produce phospho-specific yet context-independent antibodies were borne out by the Heffetz attempts: despite the fact that such method had previously produced an anti-phosphotyrosine antibody with the desired specificity, it largely failed to work for phosphothreonine. (*See* discussion in '364 specification at p. 5). Heffetz itself assigned their failure to differences in the size and immunogenicity of the bulkier phosphotyrosine residue as compared to phosphoserine and phosphothreonine (*i.e.* the hapten-carrier approach was unsuitable because these residues were too small alone to be "seen" by the immune response).

22. The prior art failures discussed in the Background of the '364 specification merely confirm that hapten-carrier techniques were not broadly suitable for production of context-independent antibodies against phosphorylated motifs. These failures are not relevant to the entirely different method of anti-peptide antibody production, which, as discussed at length above, is very predictable and useful for producing antibodies against phosphorylated peptide sequences or sites. The novel class of antibodies described in detail in the '364 specification are not produced by the hapten-carrier approach; rather, they are produced by a modified anti-peptide approach. Indeed, the Background of the '364 specification clearly describes the failure of the prior art hapten-carrier approach, as well as the shortcomings of the standard anti-peptide production methods, in focusing on the unmet need for a new class of context-independent antibodies that is solved by the invention. Accordingly, the single failed attempt reported in Heffetz is not relevant to the state of the art of anti-peptide antibody production, and to the genus of novel antibodies described and claimed in the '364 application. Indeed, where Heffetz failed, the present inventors succeeded by producing a phospho-threonine-specific, context-independent antibody (see Example I).

23. I further understand that the Examiner is concerned that no drawing or structural formulas for the presently claimed class of antibodies are provided in the '364 specification. Antibodies (unlike other biological molecules such as DNA, or chemical compounds) are rarely, if ever, described by their structure or chemical formula. Rather, antibodies are defined to those of skill in the art by their binding specificity, because the biological purpose (and usefulness) of an antibody is to bind its target. The structural features of antibodies are well known in the art, and the close structure-function correlation of antibodies has long been established. Accordingly, those of skill in the art of antibody production, characterization, and use recognize the possession of an antibody (or genus of antibodies) by a description of its binding specificity and characteristics. No drawing or structural or chemical formula is necessary. Here, the specificity of the claimed motif-specific, context-independent antibodies, which specifically bind a kinase consensus substrate motif or protein-protein binding motif comprising at least one phosphorylated amino acid, and its context-independent binding characteristic, are adequately described in the '364 specification given the level

of knowledge in the advanced art of antibody production and motifs relevant to signal transduction.

24. I further understand that the Examiner has taken the position that the claimed genus of motif-specific, context-independent antibodies (see claim 28) is not novel over the disclosure of Tani *et al.*, U.S. Patent No. 6,001,580, "Method for Assaying ERK2 MAP Kinase," issued December 14, 1999, ans/or Strulovici *et al.*, U.S. Patent No. 5,759,787, "Kinase Assay," issued June 2, 1998. I have reviewed both of these references. The Examiner's assertion is incorrect, as neither of these references discloses an antibody having the binding characteristics or specificity of the presently claimed class of antibodies (*see* claim 28). The novel class of antibodies presently claimed in the '364 application are context-independent antibodies that are specifically bind a recurring phosphorylated kinase consensus substrate motif or phosphorylated protein-protein binding motif in multiple different peptides or proteins in which the motif recurs. In contrast, the antibodies disclosed in Tani and Strulovici, respectively, are no more than traditional phosphorylation-site specific antibodies, each of which merely binds a unique epitope present in a particular protein. As discussed above, site-specific antibodies, such as those disclosed in Tani and Strulovici, are produced by the standard anti-peptide methodology (*see* Czernik, etc. discussed above) and are designed to bind non-recurring, unique epitopes in one particular target protein. The disclosed antibodies do not bind recurring phosphorylated kinase consensus substrate motifs or protein-protein binding motifs. The disclosed antibodies are not context-independent because they do not bind multiple different proteins or peptides in which the motif recurs. This shortcoming of prior art site-specific antibodies (like those of Tani and Strulovici) is discussed in the Background of the '364 specification, and is part of the problem solved by the invention, which provides a novel class of motif-specific, context-independent antibodies.

25. The antibody disclosed in Tani is a traditional site-specific anti-peptide antibody that binds the kinase ERK1, and its homologue ERK2, only when phosphorylated at a particular epitope. This antibody was generated by standard anti-peptide antibody methods (described in Czernik *et al, see supra.*), using a 12 amino acid long synthetic phospho-peptide, His-Thr-Gly-Phe-Leu-Thr*-Glu-Tyr*-Val-Ala-Thr-Arg (*=phosphorylated residue) as an antigen. This sequence corresponds to a unique epitope present in ERK1 kinase (at residues 197-208; see Fig. 6 in Tani) and its homologue ERK2 kinase (at residues 180-191; see Fig. 11 in Tani). Tani reports that this epitope was specifically chosen because it was known to be identical in both homologues of ERK kinase. *See* Tani *at* column 10, lines 50-65; see also Example 6, column 24. Tani does not disclose that this epitope recurs in any other proteins than the homologues, ERK1 and ERK2, which are essentially the same protein (it is stated that these two MAP kinase species are highly homologous (84.7%), and have never been shown to be different in either function or activity (*See* Tani at column 1, lines 26-

34)). Tani does not disclose that the epitope bound by this antibody is a kinase consensus substrate motif or protein-protein binding motif that recurs in other proteins. Tani does not disclose that this antibody is capable of binding the epitope against which it was raised in any proteins other ERK1/2. Accordingly, the site-specific antibody described in Tani is different from, and does not have the binding specificity or characteristics of the presently claimed class of motif-specific, context-independent antibodies.

26. Strulovici discloses a commercially available anti-GFAP (glial fibrillary acidic protein) antibody, YC10, to detect phosphorylation of a substrate peptide (RRRVTSAARRS, peptide #2) by PKC or PKA kinase; the substrate peptide corresponds to the GFAP epitope (residues 7-12 of GFAP) that is phosphorylated *in vivo* by these kinases. (*See* YC10 Antibody Product Data Sheet (Cat. No. NBA-115), Stressgen Bioreagents, www.stressgen.com). This antibody is a typical site-specific antibody that binds only GFAP when phosphorylated at serine 7, and was produced by standard methods (*see* Czernik) using the following 11 residue peptide as an antigen: R-R-R-V-T-phosphoSer-A-A-R-R-phosphoSer (*See id.*). GFAP is an intermediate-filament, or structural protein, that is a marker of astrocyte cell maturation. (*See id.*) Strulovici discloses that this antibody binds the target phosphorylation site in GFAP, the protein for which it is intended to be specific (see Strulovici at column 6, line 8). Strulovici does not disclose that the epitope bound by the YC10 antibody is a kinase consensus substrate motif or protein-protein binding motif recurring in other proteins (in contrast, it merely states that the GFAP site for which the YC10 antibody is specific is phosphorylatable by PKA or PKC). Strulovici does not disclose that the YC10 antibody is capable of binding the target epitope in any proteins other GFAP. Accordingly, the YC10 site-specific antibody described in Strulovici is different from, and does not have the binding specificity and characteristics of the presently claimed class of motif-specific, context-independent antibodies.

27. Strulovici also discloses the use of a monoclonal antibody, MPM-2, that binds a phosphorylated epitope in mitotic proteins, but states that the exact epitope it binds is *not known*. *See* Strulovici column 8, lines 49-53. Strulovici describes using this antibody to detect the phosphorylation, by CAMK II kinase, of a 39 amino acid peptide (peptide #4, see column 6, lines 13-15) that corresponds to a phosphorylation site in RIP (see column 6, line 15), itself a kinase involved in apoptosis (*see* Hsu *et al.* (1996), cited in Strulovici). Strulovici does not disclose that MPM-2 is specific for a phosphorylated kinase consensus substrate motif or protein-protein binding motif that recurs in other proteins. Indeed, Strulovici notes the binding specificity of this antibody is unknown. Strulovici does not disclose that the MPM-2 antibody is capable of binding any proteins or peptides other than the 39 amino acid peptide corresponding to a RIP phosphorylation epitope. Accordingly, the site-specific MPM-2 antibody described in Strulovici is different from, and does not

have the binding specificity and characteristics of the presently claimed class of motif-specific, context-independent antibodies.

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: May 11, 2004

Roberto D. Polakiewicz, Ph.D.
Tel: (978) 867-2369

TABLE II (*continued*)

Protein kinase	S:T ratio[a]	Motif[b]	Frequency
Tyrosine kinase[j]			
EGF-receptor kinase	Tyrosine	XE/DY*X	7/14
		XE/DY*I/L/V	5/14

[a] S:T ratio is for the total number of phosphorylation sites.
[b] Asterisks indicate the phosphorylated residue. Specificity determinants are shown in bold type.
[c] Three of 20 phosphorylation sites for calmodulin-dependent protein kinase II are on threonine residues, including both autophosphorylation sites.
[d] Assuming phosphorylation of Ser-3 in glycogen synthase directs Ser-7 phosphorylation.
[e] Eighteen of 30 Casein kinase II phosphorylation site sequences contain 3 consecutive acidic residues following the phosphorylated residue.
[f] cAMP-dependent protein kinase motifs and frequency data are derived from O. Zetterqvist, U. Ragnarsson, and L. Engström, *in* "Peptides and Protein Phosphorylation" (B. E. Kemp, ed.), p. 171. Uniscience CRC Press, Boca Raton, Florida, 1990, and from Table I in this chapter. The most striking feature of the cAMP-dependent protein kinase phosphorylation site sequences is the variability, with less than one-third corresponding to the **RRXS*** motif.
[g] The only autophosphorylation site included was Thr-58. Autophosphorylation at Ser-50, Ser-72,and Thr-84 occurs only following activation with cAMP.
[h] Assuming sequential phosphorylation of glycogen synthase and protein phosphatase-1 G subunit by glycogen synthase kinase-3.
[i] Twenty-three of 37 protein kinase C phosphorylation site sequences contain an adjacent hydrophobic residue on the COOH-terminal side of the phosphorylated residue.
[j] Apart from the EGF-receptor kinase, the tyrosine kinase substrate sequences do not reveal consensus recognition motifs. Relatively few phosphorylation site sequences are known for exogenous proteins and autophosphorylation sites may not reflect the specificity determinants required in substrates. A more informative indication of likely phosphorylation site arrangements may be drawn from this volume [10].

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TABLE I (*continued*)

Protein kinase	Phosphorylation site sequence	Protein	Refs.[b]
$pp60^{c\text{-}src}$ and/or unknown kinase(s)	F_{520} T S T E P Q Y* Q P G E N L	$pp60^{c\text{-}src}$	61, 66, 143
$pp60^{v\text{-}src}$	R_{409} L I E D N E Y* T A R Q G A K	Autophosphorylation	61
	S_{336} G G K G G S Y* S Q A A C S D	HLA-B7 (α chain)#	61
	S_{290} D R K G G S Y* S Q A A S S D	HLA-A2 (α chain)#	61
	H_{16} S T P P S A Y* G S V K A Y T	Lipocortin II (p36, calpactin I heavy chain)	61, 62
$p90^{gag\text{-}yes}$	R_{417} L I E D N E Y* T A R Q G A K	Autophosphorylation# (residue numbers based on human sequence)	61
$p56^{lck}$	R_{387} L I E D N E Y* T A R E G A K	Autophosphorylation	61
$p140^{gag\text{-}fps}$	R_{417} Q E E D G V Y* A S T G G M K	Autophosphorylation	61
	K_{231} Q V V E S A Y* E V I R L K G	Lactate dehydrogenase# (residue numbers based on chicken sequence)	61
	S_{36} G A S T G I Y* E A L E L R	Enolase# (residue numbers based on human sequence)	61
$p110^{gag\text{-}fes}$	R_{493} E A A D G I Y* A A S G G L R	Autophosphorylation	61, 149
$p85^{gag\text{-}fes}$	R_{361} E E A D G V Y* A A S G G L R	Autophosphorylation	61, 149
$p120^{gag\text{-}abl}$	R_{386} L M T G D T Y* T A H A G A G	Autophosphorylation# (residue numbers based on human sequence)	61
Endogenous kinase (?p40)	M_1 E E L Q D D Y* E D D M E E N	Band 3	61, 74, 75
p40	E_{669} E D G E R Y* D E D E E	Glycogen synthase (residue numbers based on human muscle sequence)	74, 150
Unknown kinase(s)	F_{498} T A T E G Q Y* Q P Q P	$p56^{lck}$	143
Unknown kinase	K_{36} K R K S* G N S R E R	Avian retrovirus nucleocapsid protein (pp12)	77
Unknown kinase	K_9 I G E G T Y* G V V Y K A R H K	$cdc2^+$ (pp34)	106

[a] #, Residue numbers obtained from SWISSPROT protein data base; (P) denotes a phosphorylated residue which acts as a substrate specificity determinant.

[b] References for Tables I and II are combined at the end of the chapter.

[c] The phosphorylation sites for the β-adrenergic receptor kinase are inferred from mutagenesis studies.[50]

[d] Artificial protein substrates have not been included for casein kinase II.

[e] Only selected phosphorylation sites for cAMP-dependent protein kinase and protein kinase C have been included to illustrate the various recognition motifs.

[f] P. J. Robinson (unpublished result, 1990).

[g] Although the growth-associated H1 histone kinases appear to require proline residues for substrate recognition, so do a number of other kinases, including glycogen synthase kinase-3, growth factor-regulated kinase, mitogen-activated protein kinase, proline-directed protein kinase, and sperm-specific histone kinase.

[h] Threonine residues 340 and 342 are assumed sites based on stoichiometry of rhodopsin phosphorylation.

[i] Some doubt exists as to whether tyrosine phosphorylation of the various tyrosine kinases is due to autophosphorylation *in vivo*.

[j] Residue numbers for protein phosphatase-1 G subunit obtained from P. Tang, J. Bondor, and A. A. DePaoli-Roach (personal communication, 1990).

TABLE II

CONSENSUS PHOSPHORYLATION SITES: SPECIFICITY MOTIFS FOR PROTEIN KINASES

Protein kinase	S:T ratio[a]	Motif[b]	Frequency
Calmodulin-dependent protein kinase II	15:5	**XRXXS*/T***	13/20[c]
		XRXXS*/T*V	6/20
Casein kinase I	8:1	**S(P)XXS*/T***	5/9[d]
Casein kinase II	28:2	**S*/T*XXEX**	23/30
		S*/T*XXDX	3/20[e]
cAMP-dependent protein kinase[f]	40:6	**RXS***	21/46
		RRXS*	12/46
		RXXS*	11/46
		KRXXS*	2/46
cGMP-dependent protein kinase	7:3[g]	**R/KXS*/T***	9/10
		R/KXXS*/T*	8/10
		R/KR/KXS*/T*	7/10
		R/KXXXS*/T*	5/10
		S*/T*XR/K	2/10
Glycogen synthase kinase-3	10:2	**S*XXXS(P)**	6/12[h]
Growth-associated histone H1 kinase (MPF, $cdc2^+$/CDC28 protein kinases)	7:8	**S*/T*PXK/R**	6/15
		K/RS*/T*P	5/15
		S*/T*PK/R	4/15
Phosphorylase kinase	3:0	**K/RXXS*V/I**	3/3
Protein kinase C	31:6	**S*/T*XK/R**	20/37[i]
		K/RXXS*/T*	13/37
		K/RXXS*/T*XK/R	7/37
		K/RXS*/T*	10/37
		K/RXS*/T*XK/R	6/37

(*continued*)

Table III Examples of Motifs for Ser/Thr Protein Kinases[a]

Kinase	Motif	Peptide substrate	K_m (μM)
PKA	RRXS	LRRASLG	4.5
		KKLTRRASFSAQ	4.3
PKG	RXXSR	RKRSRAE	29
PKC	RXXS or SXR	PLSRTLSVAAKK	4
		QKRPSQRSKYL	7
		YQRRQRKSRRTI	24
		VRKRTLRRL	48
CK2[b]	SXXE/D	RRRDDDSDDD	60
		RRREEESEEE	180
CaMKII[b]	RXXSV	PLRRTLSVAA	3.5
SmMLCK	KKRXXRXXS	KKRAARATSNVFA	7.5

[a] Derived from autophosphorylation sites and known protein substrates. Data adapted from Ref. 102.

[b] Ck2: casein kinase 2; SmMLCK: smooth muscle myosin light chain kinase

of the protein substrates or associate molecules to Src via the Src SH3 or SH2 domains may facilitate the phosphorylation of these substrates. However, there are many substrates that do not interact directly or indirectly with the SH2 or SH3 domains of Src. What then are the factors that determine the substrate specificities of PTK? Attempts have been made to use synthetic peptides to elucidate the substrate specificities of various PTKs as recognized by the catalytic domain.

Peptide Substrates for PTK

The substrate recognition sites for protein Ser/Thr kinases can generally be defined by a linear peptide sequence. For example, in PKA, the RR_S/T peptide motif has been elucidated as the substrate recognition motif. This motif is present in several known PKA substrates such as pyruvate kinase, phosphorylase kinase α-chain, and glycogen synthase.[100–102] A heptapeptide (LRRASLG or Kemptide) derived from this specific motif has a K_m value of 4.5 μM for PKA,[101] which is close to the K_m values of natural protein substrates for PKA. This suggests that the motif RR_S/T is essential and sufficient for substrate recognition and phosphorylation by PKA. Basic amino acids (R or K) are commonly found around phosphorylation sites for many Ser/Thr kinases. Table III lists the published motifs for some protein Ser/Thr kinases.

In contrast to the well-defined linear peptide substrate motifs recognized by specific Ser/Thr kinases, few peptide motifs have been identified for PTK. Peptides derived from the autophosphorylation site of various PTKs have been used for the elucidation of structural requirements for enzyme recognition, but these synthetic peptides are found to be indiscriminately phosphorylated by various PTKs.[103–107] In addition to the lack of specificity, these peptide substrates, with few exceptions, are inefficient with K_ms in the mM range.[104,108] A common feature of several autophosphorylation sites of many PTKs is the presence of multiple acidic residues on the amino terminal side of the tyrosine residue.[100] Donella-Deana and his co-workers[109] attempted to define substrate specificity of three PTKs isolated from spleen (TPK-I, TPK-IIB, and TPK-III) by systematically studying the phosphorylation kinetics of 20 synthetic peptides (dimer to hexamer) with varying number of Glu and Ala flanking a Tyr residue. Some differential specificities were detected. However, except for EEEEEY and YEEEEE peptides, all the peptides were rather inefficient with a K_m of 0.5–16 mM. Interestingly, both EEEEEY and YEEEEE peptides had a K_m of 130 μM but a different V_{max} for TPK-IIB PTK. This same enzyme was able to phosphorylate EDNEYTA (Tyr-416 of Src) and EPQYQPA (Tyr-527 of Src) peptides with a K_m of 58 μM and 16.7 mM, respectively.[110] Based on the autophosphorylation site of Src family enzymes (EDNEYTA), Ruzza et al.[108] designed and synthesized 7 peptide analogues [H-EDNEYTA-OH, Ac-EDNEYTA-NH_2, H-EDNEYTA-NH_2, c(EDNEYTA), H-(EDNEYTA)$_2$-OH, Ac-(EDNEYTA)$_2$-NH_2, and c(EDNEYTA)$_2$], and tested their ability as substrate as well as inhibitor for Lyn. They determined that the linear dimeric or cyclic dimeric peptide analogues, when compared to the linear monomeric peptide, were 2–6-fold more efficient as a substrate for Lyn. Peptide H-(EDNEYTA)$_2$-OH and c(EDNEYTA)$_2$ both had a K_m of 20 μM. McMurray et

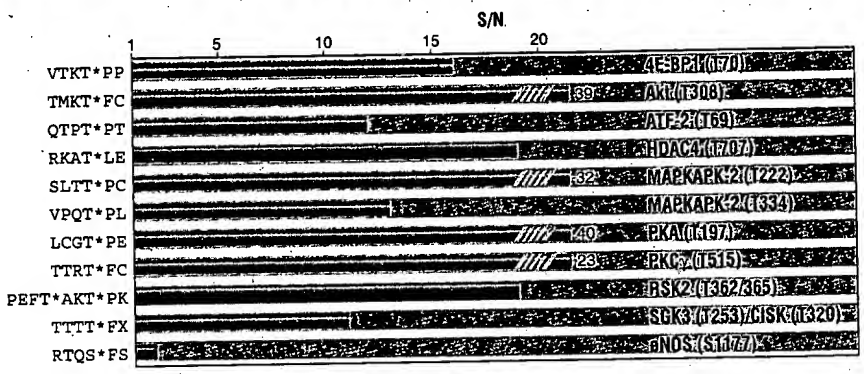
Phospho-Threonine (42H4) Monoclonal Antibody

#9386

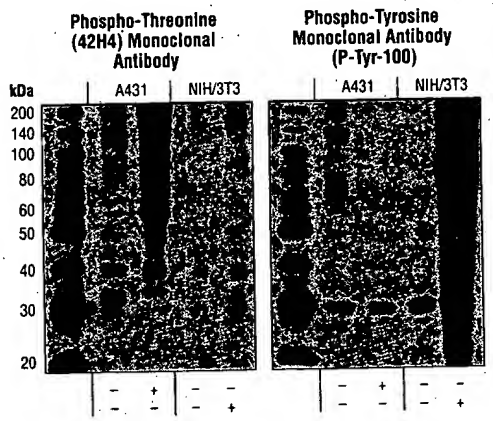
Applications	Species Cross-Reactivity	Source	Isotype	
W, IP, D	All	Mouse	IgM	#9386 S 100 µl (10 Western blots) $195 #9386 L 300 µl (30 Western blots) $475

Specificity/Sensitivity: Phospho-Threonine (42H4) Monoclonal Antibody binds phosphorylated threonine residues in a manner largely independent of the surrounding amino acid sequence. The antibody is phospho-specific but does not cross-react with phospho-serine-containing sequences. It does show slight cross-reactivity with a few phospho-tyrosine-containing peptides. By ELISA it recognizes a wide variety of threonine-phosphorylated peptides. (Patented, U.S. No. 6,441,140 and Patents Pending.)

Source/Purification: Monoclonal antibody is produced by immunizing mice with phospho-Thr-containing peptides (KLH coupled). Antibody is supplied in HEPES buffer with 50% glycerol and less than 0.02% sodium azide.



Phospho-Threonine (42H4) Monoclonal Antibody DELFIA® assay: Signal-to-noise ratio of phospho- versus nonphospho-peptides. (T denotes phosphorylated threonine.) (DELFIA® is a registered trademark of PerkinElmer, Inc.)*



◀ *Western blot analysis of extracts from A431 cells untreated or treated with calyculin A, a threonine phosphatase inhibitor, or extracts from NIH/3T3 cells untreated or treated with pervanadate, a tyrosine phosphatase inhibitor, using Phospho-Threonine (42H4) Monoclonal Antibody (left) or Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) #9411 (right).*

Motif-Specific Antibodies from Cell Signaling Technology

EPITOPE	ANTIBODY
Y*-X-X-M	Phospho-(Tyr) p85 PI3K Binding Motif Antibody (#3821)
X-T*-P-X	Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101) (#9391)
X-T*-X-R/K	Phospho-Threonine-X-Arginine Antibody (#2351)
X-T*-F-C-G-T-X	Phospho-(Thr) PDK1 Substrate Antibody (#2291)
X-R/K-X-X-S*-X-P	Phospho-(Ser) 14-3-3 Binding Motif Antibody (#9601)
X-R/K-X-X-S*-X-P	Phospho-(Ser) 14-3-3 Binding Motif (4E2) Monoclonal Antibody (#9606)
R-X-Y/F-X-S*-X-X	Phospho-(Ser) Arg-X-Tyr/Phe-X-pSer Motif Antibody (#2981)
X-X-R/K-X-S*-Hyd-R/K	Phospho-(Ser) PKC Substrate Antibody (#2261)
R/K-X-R/K-X-X-S*/T*-X-X	Phospho-(Ser/Thr) AKT Substrate Antibody (#9611)
R-X-X-T*-X-X/R-R-X-S*-X-X	Phospho-(Ser/Thr) PKA Substrate Antibody (#9621)
Hyd-S*/T*-Q-X	Phospho-(Ser/Thr) ATM/ATR Substrate Antibody (#2851)
Y/F/W-S*/T*-F	Phospho-(Ser/Thr) Phe Antibody (#9631)
F-X-X-F/Y-S*/T*-F/Y	Phospho-(Ser/Thr) PDK1 Docking Motif (18A2) Monoclonal Antibody (#9634)